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Effect of Protease EPg222 Obtained from *Penicillium chrysogenum* Isolated from Dry-Cured Ham in Pieces of Pork Loins

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The fungal protease EPg222 obtained from *Penicillium chrysogenum* Pg222 isolated from dry-cured ham, was assayed for proteolytic activity in a meat model system based on sterile pieces of pork loins for 32 days. Treated samples showed a significative reduction of total high ionic strength-soluble proteins during the incubation period, as compared with a control incubated without enzyme, both on the surface and in the depth. SDS-PAGE analysis of this protein fraction showed higher hydrolysis of the main myofibrillar proteins H-meromyosin, actin, and tropomyosin in treated samples. Non-protein and amino acidic nitrogen were detected in higher amounts in enzyme-added than in control pieces of loins, both on the surface and in the depth. Thus, addition of enzyme EPg222 to whole pieces of meat results in an increase of protein hydrolysis. The effect of this enzyme could be of great interest for stimulating proteolysis in whole dry-cured meat pieces.

KEYWORDS: ***Penicillium chrysogenum*; protease; proteolysis; dry-cured meat products**

INTRODUCTION

Dry-cured meat products are appreciated for their typical flavor, but the development of the different biochemical reactions requires in some cases long ripening times. During ripening proteolysis takes place, yielding peptides and free amino acids, which are involved in taste and flavor development (*1*−*3*). Meat protein hydrolysis is mainly catalyzed by endogenous enzymes, such as cathepsins (*4*) and trypsin-like peptidases (*5*). However, curing agents (*6*) and the free amino acid released (*7*) produce a strong inhibitory effect on the muscle peptidases. On the other hand, the microbial population growing mainly on the surface of these products shows aminopeptidase activity (*8*, *9*) not affected by the level of amino acids present. This activity contributes to protein hydrolysis in dry-cured meat products, mainly in the latter stages of the ripening process, when muscle peptidase activity is reduced. Since, in some cases, microbial growth on dry-cured meat products is not desirable, proteolysis could be promoted using purified microbial proteases. Thus, an adequate level of proteolysis could be reached early, which could allow a shorter ripening process without affecting the flavor of the ripened products.

Several microbial proteases have been assayed to accelerate proteolysis in minced dry fermented sausages (*10*−*13*). However, very little work has been done with proteases in whole ripened pieces such as dry-cured ham and ripened loins. In these products the tissue structure, including the different layers of connective tissue and intramuscular fat, may limit access of the enzymes to deep tissues. One of the main obstacles in determining the effect of the proteases on the ripening process of dry-cured whole pieces is the lack of a sterile control. This difficulty could be overcome using a meat model system based on sterile pieces of pork loins incubated under aseptic conditions.

The use of proteases obtained from microorganisms isolated from dry-cured meat products could be more appropriate than other ones, since the proteases from the dry-cured meat might be more suited and adapted to function during the ripening process. Protease EPg222 purified from *Penicillium chrysogenum* Pg222 isolated from dry-cured ham has shown high proteolytic activity against myofibrillar proteins at those conditions of temperature, pH, and NaCl concentration of dry-cured meat products (*14*).

The aim of this work has been to investigate the proteolytic activity of the protease EPg222 in pieces of pork loins incubated at the conditions of temperature and NaCl concentration of dry-cured meat products.

MATERIAL AND METHODS

Extracelullar Enzyme. EPg222 is a protease obtained from *P. chrysogenum* Pg222 isolated from dry-cured ham. The optimum working conditions of this protease are 45−55 °C, pH 6, and 0.25 M NaCl (*14*).

Preparation of Pieces of Loins and Enzyme Treatment. Pork loins were removed from carcasses 15 min after slaughter, and the external surface was sterilized (*15*). Then, loins were cut into 40 small pieces of 150−200 g. Each piece was placed on sterile stomacher bags, with

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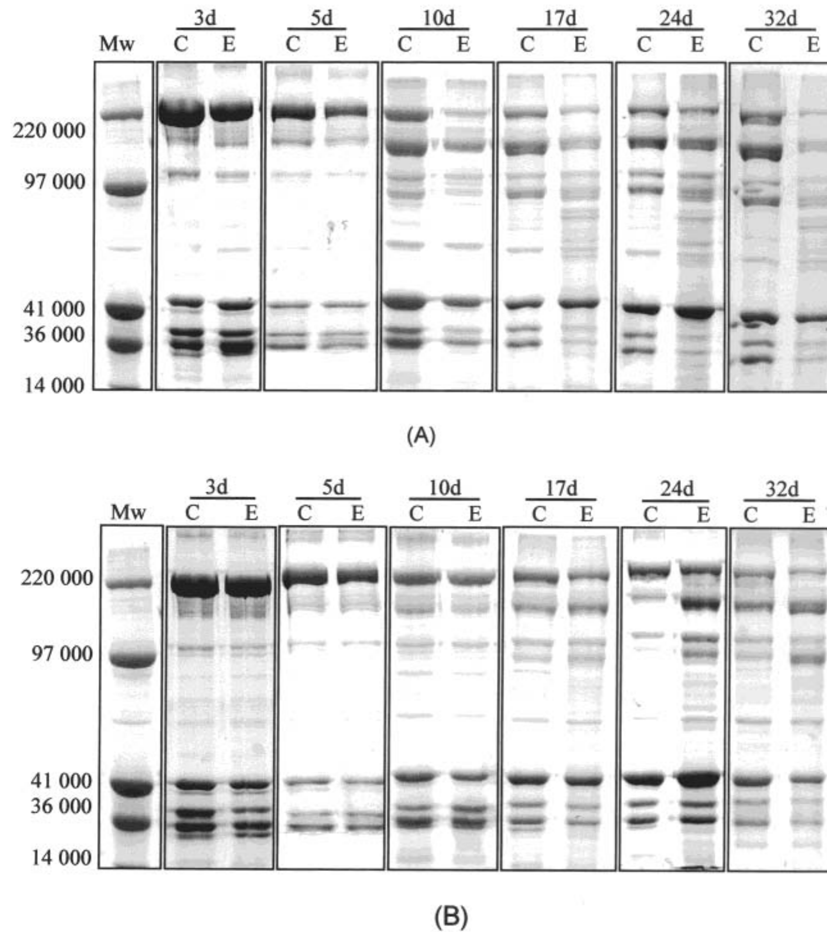

Figure 1. SDS-7.5% PAGE of high ionic strength-soluble proteins of enzyme-treated (E) and control (C) pieces of pork loins, on the surface (A) and in the depth (B).

the addition of 5% NaCl, and left for 48 h at 4 °C. After salting, 20 pieces of loins were placed in separated sterile stomacher bags and added to 30 mL of a sterile solution containing 4 mg mL^{-1} of chloramphenicol, 1 mg mL^{-1} of cicloheximide, and 0.35 mg of enzyme EPg222. The remaining pieces of loins were added to 30 mL of the former solution without enzyme (untreated control samples). Treated and untreated samples were incubated at 20 °C for 32 days in sterile conditions. Samples were taken at 3, 5, 10, 17, 24, and 32 days of incubation, and three samples of the treated and control batches were analyzed at each time. For analysis the first 10 mm of the pieces of loins were considered as surface and the inner 10 mm as the depth, except for the measurement of hardness that was carried out over the whole pieces of pork loins.

Chemical Analysis. *Moisture.* Moisture content was determined over 5 g samples by the ISO-1442.

Extraction and Quantification of Low (Sarcoplasmic) and High (Myofibrillar) Ionic Strength-Soluble Proteins. Low ionic strength-soluble proteins were extracted from 2 g samples with 40 mL of 0.03 M, pH 7.4, sodium phosphate buffer (*16*). The samples were homogenized in a Sorvall omnimixer (Omni Corp. International Instruments, Waterbury, CT). The extracts were centrifuged at 8000*g* for 15 min at 4 °C, and the supernatants were filtered through a 0.45 μm filter. Myofibrillar proteins were extracted from the resultant pellet with 40 mL of 1.1 M potassium iodide + 0.1 M sodium phosphate, pH 7.4, buffer following the steps indicated for the above extraction. The concentration of protein was determined following the Bradford method (*17*) using 100 μL of the low and high ionic strength-soluble protein extracts.

Analysis by SDS−PAGE of Low and High Ionic Strength-Soluble Proteins. For denaturation of low and high ionic strength-soluble protein fractions, 2 μL of extract was denatured by boiling for 5 min in 0.0625 M Tris-HCl buffer, pH 6.8, with 20% glycerol, 2% SDS, and 5% 2-β-mercaptoethanol. Then, proteins were characterized by 7.5% SDS-PAGE (*18*). Density and molecular weight of the protein bands were measured using a Kodak Digital Science software package (Kodak Digital Science, Rochester, NY). Myosin (Mr, 220 000), phosphorylase B (Mr, 97 000), creatin kinase (Mr, 41 000), glyceraldehide phosphate dehydrogenase (Mr, 36 000), and myoglobin (Mr, 14 000) (Sigma Chemical Co., St. Louis, MO) were used as standards.

Nonprotein Nitrogen. Non-protein nitrogen (NPN) was determined by the Nessler method using 4 g of sample after protein precipitation with 0.6 M perchloric acid, as described by De Ketalere et al. (*19*).

Amino Acid Nitrogen. Amino acid nitrogen (AN) was determined from the 0.6 M perchloric acid protein precipitation fraction after peptide precipitation with 10% sulfosalicilic acid, according to Martín et al. (*20*).

Hardness Analysis. Hardness of the samples was measured at room temperature, using TA-XT2 texture analyzer with XT-RA dimension software (Stable Micro Systems, Godalming, U.K.). Cubic samples obtained from whole pieces of pork loins of about 4 × 4 × 2.5 cm were compressed twice to 25% of their original height with a compression platen of 40 mm in diameter. Force−time curves were developed at a cross-head of 0.6 mm/s, and the recording speed was 2 mm/s. Hardness was evaluated and defined by peak force during the first compression cycle.

Statistical Analysis. Statistical analysis of the data was carried out using one-way analysis of variance, and the mean was separated by Tukey's honest significant difference test using SPSS for Windows, 10.0. (SPSS Inc., Chicago, IL).

RESULTS

Total high ionic strength-soluble proteins showed significative ($P < 0.01$) decreases during the 32 days of incubation in control

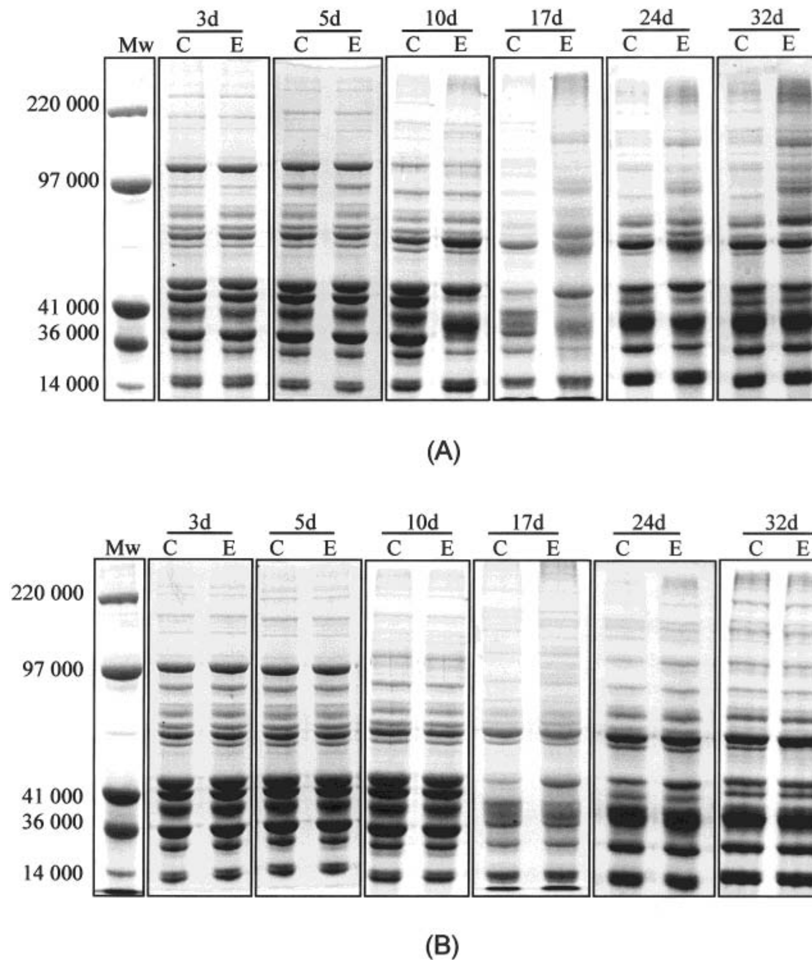

Figure 2. SDS-7.5% PAGE of low ionic strength-soluble proteins of enzyme-treated (E) and control (C) pieces of pork loins at different days (d) of incubation, on the surface (A) and in the depth (B).

Table 1. Total High Ionic Strength-Soluble Proteins of Enzyme-Treated (E) and Control (C) Pieces of Loins at Different Incubation Times[a]

days of incubation	mg of protein/(g of dry matter)			
	surface		depth	
	C	E	C	E
3	$118.22 \pm 4.54^{a}_{1}$	$121.55 \pm 6.65^{a}_{1}$	$136.07 \pm 4.58^{a}_{1}$	$131.14 \pm 7.62^{a}_{1}$
5	$102.80 \pm 3.70^{a}_{1}$	$95.40 \pm 2.91^{a}_{2}$	$99.79 \pm 2.61^{a}_{2}$	$113.89 \pm 11.82^{a}_{1,2}$
10	$88.18 \pm 11.09^{a}_{1,2}$	$84.69 \pm 8.09^{a}_{2}$	$96.03 \pm 0.71^{a}_{1,2}$	$114.21 \pm 7.92^{a}_{1,2}$
17	$94.82 \pm 1.60^{a}_{2}$	$46.69 \pm 5.22^{b}_{3}$	$110.94 \pm 9.01^{a}_{2}$	$98.13 \pm 4.60^{a}_{2}$
24	$99.26 \pm 4.74^{a}_{2}$	$38.90 \pm 10.48^{b}_{3}$	$105.07 \pm 6.13^{a}_{2}$	$94.62 \pm 4.65^{a}_{2}$
32	$97.16 \pm 4.27^{a}_{2}$	$33.28 \pm 2.87^{b}_{3}$	$110.01 \pm 7.17^{a}_{2}$	$75.48 \pm 6.20^{b}_{3}$

[a] For a given batch (row), values followed by different letters as superscript are significantly different ($P < 0.01$) from its control. For a given incubation time (column), values with different numbers as subscript are significantly different ($P < 0.01$).

and enzyme-treated samples (Table 1). Treated samples showed a significant reduction of this protein fraction at 17, 24, and 32 days on the surface and at 32 days in the depth, as compared with the control (Table 1).

Analysis of SDS-PAGE of high ionic strength-soluble proteins revealed on the surface higher protein hydrolysis after 10 days of ripening in enzyme-treated than in control samples. At this sampling time the main bands of molecular weight 220 000 (H-meromyosin) and 46 000 (actin) were significantly ($P < 0.01$) reduced in 46 and 38%, respectively, as compared with the control. No changes were observed in the depth at this time (Figure 1). However, at 17 days of incubation the intensity of the main bands in the treated samples were significantly reduced as compared to the control, both on the surface and in the depth. Thus, at this sampling time H-meromyosin showed in treated samples a reduction of 77.29% on the surface and 44.3% in the depth. Reductions of at least 20% on the surface and 10% in the depth were observed in other myofibrillar protein bands such as molecular weight 46 000 (actin), 39 000 (troponine I), and 37 000 (tropomyosin) in enzyme-treated samples. No deeper reduction of the intensity of bands was observed on the surface of treated samples at 24 and 32 days. However, in the depth further reductions in the bands intensity were observed. Thus, at 32 days in the depth, the main bands of molecular weight 220 000, 46 000, 39 000, and 37 000 showed significant reductions of 70, 46, 18, and 20%, respectively, as compared to the control.

At 17 days incubation time, new bands of approximately 89 000, 87 000, 76 000, and 70 000 were detected only at the surface of treated samples. The bands of 89 000, 87 000, and 76 000 are also detected in the depth at 32 days of incubation.

Total low ionic strength-soluble proteins decreased ($P < 0.01$) through the incubation process in both treated and control samples (Table 2). Treated samples showed a significantly lower amount than the control at day 3 on the surface and at day 5 in the depth. However, in the depth at 17 and 24 days enzyme-treated samples showed a significantly higher amount of this protein fraction than the control.

SDS-PAGE analysis of the low strength-soluble protein fraction revealed only slight differences between control and enzyme-treated samples (Figure 2). At 10, 17, 24, and 32 days sampling, treated samples showed on the surface significant

Table 2. Total Low Ionic Strength-Soluble Proteins of Enzyme-Treated (E) and Control (C) Meat at Different Incubation Times[a]

days of incubation	mg of proteins/(g of dry matter)			
	surface		depth	
	C	E	C	E
3	$177.23 \pm 9.56^{a}_{1}$	$152.25 \pm 4.99^{b}_{1}$	$158.87 \pm 7.31^{a}_{1}$	$156.70 \pm 7.56^{a}_{1}$
5	$130.60 \pm 6.41^{a}_{2,3}$	$132.27 \pm 4.91^{a}_{1,2}$	$145.00 \pm 6.32^{a}_{1}$	$113.89 \pm 0.74^{b}_{2,3}$
10	$133.82 \pm 5.51^{a}_{2}$	$113.30 \pm 7.39^{a}_{2,3}$	$115.20 \pm 6.03^{a}_{2}$	$117.37 \pm 6.76^{a}_{2,3}$
17	$115.23 \pm 3.68^{a}_{2,3}$	$121.63 \pm 19.18^{a}_{2,3}$	$110.94 \pm 10.72^{a}_{2}$	$131.38 \pm 8.09^{b}_{2}$
24	$112.65 \pm 3.31^{a}_{2,3}$	$97.64 \pm 6.88^{a}_{3,4}$	$95.78 \pm 6.68^{a}_{2,3}$	$121.60 \pm 9.29^{b}_{2,3}$
32	$107.35 \pm 4.60^{a}_{3}$	$87.06 \pm 4.43^{a}_{4}$	$90.04 \pm 2.71^{a}_{3}$	$103.89 \pm 2.71^{a}_{3}$

[a] For a given batch (row), values followed by a letter are significantly different ($P < 0.01$) from its control. For a given incubation time (column), values with different numbers as subscript are significantly different ($P < 0.01$).

Table 3. Non-protein Nitrogen (NPN) of Enzyme-Treated (E) and Control (C) Pieces of Loins at Different Incubation Times[a]

days of incubation	mg of N/(g of dry matter)			
	surface		depth	
	C	E	C	E
3	$5.28 \pm 0.45^{a}_{1}$	$7.01 \pm 1.71^{a}_{1}$	$5.18 \pm 0.46^{a}_{1}$	$5.46 \pm 1.11^{a}_{1}$
5	$7.69 \pm 0.61^{a}_{1}$	$8.43 \pm 1.15^{a}_{1}$	$7.25 \pm 0.24^{a}_{1}$	$6.85 \pm 0.64^{a}_{1}$
10	$9.16 \pm 0.34^{a}_{1}$	$10.27 \pm 0.88^{a}_{1}$	$8.47 \pm 1.02^{a}_{1,2}$	$9.34 \pm 0.34^{a}_{1,2}$
17	$15.64 \pm 1.85^{a}_{2}$	$23.17 \pm 1.06^{b}_{2}$	$9.55 \pm 0.97^{a}_{1,2,3}$	$15.72 \pm 4.62^{b}_{3}$
24	$14.12 \pm 2.69^{a}_{2}$	$39.30 \pm 2.12^{b}_{3}$	$10.30 \pm 1.70^{a}_{1,2,3,4}$	$13.64 \pm 0.83^{a}_{2,3,4}$
32	$15.06 \pm 1.57^{a}_{2}$	$34.68 \pm 1.72^{b}_{4}$	$15.23 \pm 3.38^{a}_{4}$	$14.95 \pm 1.69^{a}_{3,4}$

[a] For a given batch (row), values followed by different letters as superscript are significantly different ($P < 0.01$) from its control. For a given incubation time (column), values with different numbers as subscript are significantly different ($P < 0.01$).

Table 4. Amino Acidic Nitrogen (AN) of Enzyme-Treated (E) and Control (C) Pieces of Loins at Different Incubation Times[a]

days of incubation	mg of N/(g of dry matter)			
	surface		depth	
	C	E	C	E
3	$0.51 \pm 0.06^{a}_{1}$	$1.01 \pm 0.16^{a}_{1}$	$0.56 \pm 0.16^{a}_{1}$	$0.92 \pm 0.45^{a}_{1}$
5	$0.79 \pm 0.27^{a}_{1}$	$1.31 \pm 0.12^{a}_{1,2}$	$0.69 \pm 0.08^{a}_{1}$	$1.22 \pm 0.11^{a}_{1,2}$
10	$1.09 \pm 0.11^{a}_{1,2}$	$2.11 \pm 0.29^{a}_{1,2,3}$	$1.11 \pm 0.06^{a}_{1,2}$	$1.61 \pm 0.39^{a}_{1,2}$
17	$2.76 \pm 0.18^{a}_{2,3}$	$4.87 \pm 0.33^{b}_{4}$	$1.58 \pm 0.29^{a}_{1,2}$	$2.56 \pm 0.88^{b}_{3}$
24	$3.11 \pm 0.19^{a}_{3}$	$5.90 \pm 0.96^{b}_{4,5}$	$2.43 \pm 0.07^{a}_{2}$	$3.63 \pm 0.77^{b}_{3,4}$
32	$3.41 \pm 0.49^{a}_{3}$	$7.01 \pm 1.84^{b}_{5}$	$2.55 \pm 0.23^{a}_{2}$	$4.16 \pm 0.66^{b}_{4}$

[a] For a given batch (row), values followed by a letter are significantly different ($P < 0.01$) from its control. For a given incubation time (column), values with different numbers as subscript are significantly different ($P < 0.01$).

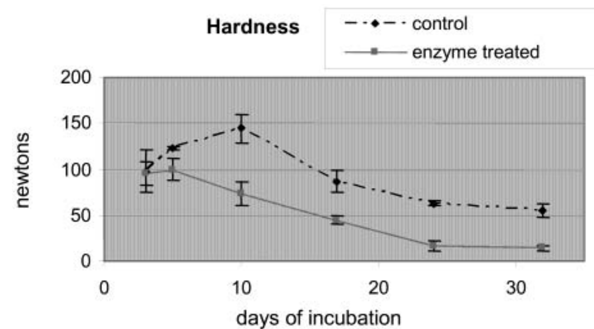

Figure 3. Hardness of enzyme-treated and control pieces of pork loins during incubation time.

reductions of the intensity of the main protein bands of 119 000, 66 000, and 28 000, as compared to the control. No significant differences were observed in the remaining main protein bands of 46 000, 37 000, and 14 000. In the depth, the only difference between treated and control samples is the disappearance of the 60 000 band at 32 days of incubation. Furthermore, at 10, 17, 24, and 32 days sampling time several protein bands of about 139 000, 121 000, 101 000, and 94 000 were only detected at the surface in treated samples (Figure 2).

The level of NPN increased ($P < 0.01$) during the incubation period in both control and enzyme-added samples (Table 3). This nitrogen fraction was significantly ($P < 0.01$) higher on the surface of the treated samples after 17 days of incubation as compared to the control. In the depth, at 17 day sampling time, the enzyme-added batch showed significantly higher values than the untreated control. However, there were no differences in this location in the next sampling times.

Table 4 shows the changes in amino acid nitrogen in both control and enzyme-added batches throughout the incubation period. A significant ($P < 0.01$) increase can be observed in the AN concentration in the analyzed batches during the incubation time. After 17 days of incubation the AN concentration was significatively higher in enzyme-added than in control samples, both on the surface and in the depth (Table 4).

Hardness analysis showed significatively lower values in treated than control samples at 10, 17, 24, and 32 days of incubation (Figure 3). At the end of the incubation period, treated samples showed a reduction of hardness of 73% as compared with control.

DISCUSSION

Analysis of total high ionic strength-soluble proteins reveals a more extensive protein hydrolysis in treated than in control samples due to the proteolytic effect of the enzyme EPg222. Furthermore, the reduction in the concentration of this protein fraction took place before on the surface than in the depth, in relation to the enzyme penetration efficiency. The hydrolysis observed in this protein fraction is higher than that reported for bacterial proteases in fermented sausages (*21*).

SDS-PAGE analysis of the strength-soluble protein fraction shows a more extensive hydrolysis of the myofibrillar proteins H-meromyosin, actin, and tropomyosin in enzyme-treated than in control samples at 10, 17, 24, and 32 days of incubation. These results confirm the observed effect of the EPg222 in the reduction of strength-soluble protein, since myofibrillar proteins are the main components of this fraction. In addition, SDS-PAGE analysis of myofibrillar proteins revealed the proteolytic effect of EPg222 at 10 day sampling, not detected by determination of total strength-soluble proteins. The effect of EPg222 over the main myofibrillar proteins confirms in meat the activity demonstrated by this enzyme against extracted meat myofibrillar proteins in vitro (*14*). High activity over H-meromyosin has also been referred for other purified fungal proteases (*22*), although no activity against actin and tropomyosin was reported.

On the other hand, the enzyme shows the ability to penetrate to the depth, despite tissue structure and intramuscular fat, since in this location myofibrillar proteins are more extensively hydrolyzed than in the control. Activity of EPg222 is also demonstrated by the release on the surface and in the depth of new bands of molecular weight ranging from 89 000 to 70 000, nondetected in control samples. Bands of similar molecular weight, mainly derived from hydrolysis of H-meromyosin, have been observed when myofibrilar proteins were incubated with EPg222 (*14*). The appearance of these degradative products seems to demonstrate that EPg222 shows endopeptidase activity. This fact could be of great interest in stimulating proteolysis in

meat products of long ripening process, in the half of ripening, when activity of tisular endopeptidases has been reduced by the effect of NaCl and other curing agents (*23*). EPg222 has shown proteolytic activity in the range of 0−3 M NaCl (*14*). In the present work this enzyme seems to be active at 5% NaCl, which is the usual NaCl concentration on the surface of dry-cured whole pieces of meat (*16*).

Analysis of total low ionic strength-soluble proteins seems to indicate an hydrolytic effect of the enzyme at the beginning of the incubation period, since the amount of this protein fraction is lower than the control. However, in the next sampling time only, there were significative differences in the depth and in some sampling time, showing higher amount of treated than of control samples. In addition, SDS-PAGE analysis of this protein fraction showed only small differences in sarcoplasmic proteins, which are the main component of this protein fraction. Thus, sarcoplasmic proteins are only slightly affected by the enzyme EPg222. This enzyme did not show activity against the sarcoplasmic protein myoglobin in vitro assays (*14*). Analysis of SDS-PAGE of low ionic strength-soluble proteins also reveals higher accumulation of peptides ranging from 109 000 to 75 000 in treated than in control samples at 10, 17, 24, and 32 days, mainly on the surface. These peptides may arise from the hydrolysis of myofibrillar proteins, especially H-meromyosin. In this protein fraction has been reported soluble peptides derived from hydrolysis of myofibrillar proteins during ripening of dry-cured meat products (*16*, *21*). At the same time that low ionic soluble peptides are being generated from the hydrolysis of myofibrillar proteins, some sarcoplasmic proteins are hydrolyzed. Thus, the final balance in low strength-soluble proteins is negligible, and differences between treated and control are not detectable when total low strength-soluble proteins are determined.

Protein hydrolysis observed in myofibrillar and sarcoplasmic proteins of treated samples, at higher levels than the control, lead to major accumulation of NPN in the former, until 2-fold more than those of the control on the surface. These results together with those of myofibrillar and sarcoplasmic proteins support an endopeptidase activity.

The evolution of AN is similar to that observed for NPN, that is,major accumulation of AN in treated samples after 17 days of ripening both on the surface and in the depth. At 32 days of ripening on the surface, the concentration of AN is 2-fold higher than the control and in the depth mean values of NA are at least 1 mg of N higher in the treated than in the control batch. Thus, addition of EPg222 to whole ripened meat leads to a higher accumulation of free amino acids, both on the surface and in the depth. This suggests that addition of endopeptidase EPg222 shows exopeptidase activity. The increase in AN observed in this work is higher than that reported in dry fermented sausages ripened with a bacterial protease (*21*). It is possible that the effect of fungal proteases adapted to the dry-curing process is higher than that of other microbial proteases. The liberation of free amino acids plays and important role in the development of dry cured meat flavor (*24*), and the presence of these compounds has been associated with such taste descriptors as spicy, beefy, sweet, bitter, and astringent (*25*). Although enzyme EPg222 could be of great interest in accelerating flavor development in dry-cured meat products, it should be noted that besides the addition of this protease, some microbial (oxidative deaminations, decarboxylations, etc.) and/or chemical (Strecker and Maillard) reactions could be necessary to yield a higher amount of flavor compounds, as has been referred to by Díaz et al. (*12*).

EPg222 decreased the hardness of pieces of pork loins, probably due to its effect on myofibrillar proteins and the collagenolytic activity reported for this enzyme (*14*). In dry-cured meat products, this effect could be of great interest in reducing the increase of hardness reported throughout the ripening process in these kinds of products due to protein denaturation (*26*, *27*).

From these results it can be deduced that it is possible to accelerate proteolysis in whole ripened pieces of meat by addition of EPg222. However, further studies should be done in dry-cured meat products to know the appropriate concentration of the enzyme to avoid an excessive hydrolysis that could have a stronger effect on softening than on flavor (*28*). This drawback is not probable, since *P. chrysogenum*, from which EPg222 was obtained, has been reported at high counts as a natural contaminant in normal dry-cured hams (*29*).

In conclusion, addition of enzyme EPg222 to pieces of loins results in an increase of hydrolysis of myofibrillar proteins on the surface and in the depth, which lead to a major accumulation of free amino acids. This enzyme could be of great interest in stimulating proteolysis in whole ripened meat pieces.

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